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Interleukin-17A increases leptin production in human bone marrow mesenchymal stem cells

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ABSTRACT

Lineage commitment of human bone marrow mesenchymal stem cells (hBM-MSCs) to adipocytes or osteoblasts has been suggested as a model system to study the relationship between type II diabetes and abnormal bone metabolism. Leptin and IL-17A inhibit adipogenesis whereas they promote osteogenesis in MSCs. Due to pathophysiologic roles of IL-17A in human metabolic diseases and bone metabolism, it was evaluated whether IL-17A-dependent inverse regulation on adipogenesis and osteogenesis was related to endogenous leptin production in hBM-MSCs. In the analysis of adiponectin and leptin secretion profiles of hBM-MSCs in response to various combinations of differentiation inducing factors, it was found that dexamethasone, a common molecule used for both adipogenesis and osteogenesis, increased leptin production in hBM-MSCs. Importantly, the level of leptin production during osteogenesis in hBM-MSCs was higher than that during adipogenesis, implicating a significant leptin production in extra-adipose tissues. IL-17A increased leptin production in hBM-MSCs and also under the condition of osteogenesis. In spite of direct inhibition on adipogenesis, IL-17A up-regulated leptin production in hBM-MSC-derived adipocytes. Anti-leptin antibody treatment partially antagonized the IL-17A dependent inhibition of adipogenesis in hBM-MSCs, suggesting a role of leptin in mediating the inverse regulation of IL-17A on osteogenesis and adipogenesis in hBM-MSCs. Therefore, the IL-17A-induced leptin production may provide a key clue to understand a molecular mechanism on the lineage commitment of hBM-MSCs into adipocytes or osteoblasts. In addition, leptin production in extra-adipose tissues like MSCs and osteoblasts should be considered in future studies on leptin-associated human diseases.

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1. Introduction

Type II diabetic patients are included in a high risk group of hip bone fracture [1,2]. However, recent studies have shown that bone mineral density of type II diabetic patients is generally higher than normal, suggesting that the quality of bone formation is degenerated in type II diabetic patients [2,3]. In human osteoporosis, fat accumulation is increased in the human osteoporotic bone marrow cavity [4,5]. The preferential differentiation of human bone marrow mesenchymal stem cells (hBM-MSCs) to adipocytes in marrow has been suggested as a critical pathologic process for age-related osteoporosis. However, the causality of the inverse relationship of hBM-MSC differentiation to adipocytes instead of osteoblasts in osteoporotic pathogenesis is still controversial [5]. In this regard, the lineage commitment of hBM-MSCs has been suggested as a model system not only to study the pathogenic progression of human metabolic syndromes like type II diabetes but also to understand bone diseases [6,7]. Currently, molecular mechanisms to regulate the fate decision of hBM-MSCs to adipocyte or osteoblast lineage pathways in disease states like type II diabetes and osteoporosis are poorly understood.

A variety of cytokines affect the lineage commitment of hBM-MSC differentiation. The inverse regulation on hMSC differentiation has been reported in functional studies with cytokines like transforming growth factor-β (TGFβ) family members [8]. For example, TGFβ and bone morphogenetic protein 3 (BMP3) can initiate and promote the early stage of osteogenesis, but it inhibits adipogenesis [8,9]. IL-17A inhibits adipogenesis in hBM-MSCs and regulates pro-inflammatory responses by increasing cellular production of IL-6, IL-8 and prostaglandin E_2 [10]. IL-17A not only promotes osteogenesis in hBM-MSCs, but also it regulates bone metabolism [11,12]. IL-17A reciprocally affects hBM-MSC differentiation into either osteoblasts or adipocytes [10–12]. Recent studies with various animal models have suggested that IL-17A is associated with metabolic diseases like diabetes and obesity [13,14]. Because of its association with the pathogenesis of metabolic diseases in bone and fat tissues, the inverse regulation of IL-17A on adipogenesis and osteogenesis in hBM-MSCs may

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provide key clues to understand the bone fracture risk of type II diabetes. In addition, leptin was reported not only to promote osteogenesis but also to inhibit adipogenesis in a conditionally immortalized human marrow stromal cell line (hMS2-12) that was established by transfecting temperature sensitive simian virus 40 large T antigen (SV40LTV) [15]. Because leptin is an adipokine that is known to be produced in adipocytes, it will be interesting to study the role of endogenously produced leptin in adipogenesis in terms of reciprocal regulation on the lineage commitment of hBM-MSCs.

To induce adipogenesis *in vitro*, hBM-MSCs are treated with dexamethasone, insulin, isobutylmethylxanthine (IBMX), and a peroxisome proliferator activating receptor (PPAR) γ agonist like troglitazone [9,10]. The osteogenic medium for hBM-MSCs includes dexamethasone, vitamin C, and β-glycerol phosphate [9,11]. Although dexamethasone is used to induce hBM-MSCs to differentiate into both osteoblasts and adipocytes, it was reported that a single dexamethasone treatment preferentially directed hBM-MSC differentiation [16]. In hBM-MSCs, dexamethasone decreased the level of IL-11, a cytokine that promotes osteogenesis while it potently inhibits adipogenesis [17,18]. The dexamethasone-induced down-regulation of IL-11 can be interpreted as a negative effect on osteogenesis in hBM-MSCs. In addition, the biological responses of hBM-MSCs to corticosteroids like dexamethasone may provide a clue to understanding the complex relationship between diabetes and osteoporosis. For instance, hypercortisolism like Cushing's syndrome is associated with both type II diabetes and bone thinning leading to rib and spine fractures [19]. However, it has been poorly evaluated in a systemic level how dexamethasone regulates hBM-MSC differentiation and whether these changes are associated with disease pathogenesis.

In the present study, in order to understand molecular mechanisms to regulate differential lineage commitment of hBM-MSCs, adiponectin and leptin production profiles during adipogenesis and osteogenesis were evaluated after hBM-MSCs were treated with various combinations of differentiation inducing factors. Interestingly, leptin was significantly up-regulated in hBM-MSCs in response to dexamethasone. Due to the fact that leptin reciprocally affects adipogenesis and osteogenesis in hBM-MSCs, it was further evaluated whether leptin was associated with the reciprocal regulation of IL-17A on hBM-MSC differentiation to osteoblasts and adipocytes.

2. Materials and methods

2.1. Cell culture and differentiation

Human bone marrow mesenchymal stem cells (hBM-MSCs) were purchased from Lonza, Inc. (Walkersville, MD, USA) and cultured as previously described [10,20]. To induce adipocyte differentiation, 10 μg/mL insulin, 100 nM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 2 μM troglitazone were added in 10% FBS/DMEM growth media. To induce osteoblast differentiation, the growth medium was supplemented with 100 nM dexamethasone, 10 mM β-glycerol phosphate, and 50 μM ascorbic acid.

2.2. Assessment of adipogenesis and osteogenesis

Adipogenesis was assessed by Oil Red O staining by a method previously described [10,20]. To visualize the nucleus, hBM-MSCs were counterstained with hematoxylin reagent for 2 min and then washed twice with H_2O. To assess osteogenesis, alkaline phosphatase activity was measured by a method previously described [16,21]. To assess mineralization, cultures were stained with silver nitrate (Von Kossa's staining) [21]. The differentiated adipocytes and osteoblasts were photographed using an Olympus IX71 inverted phase-microscope (Olympus Co., Tokyo, Japan).

2.3. Enzyme linked immunosorbant assay (ELISA) to measure cytokine production

For quantitative determination of adiponectin, leptin, and osteoprotegerin (OPG) in cell culture supernatants, Quantikine™ immunoassay kits were used (R&D Systems, Minneapolis, MN, USA). Conditioned media were centrifuged for 5 min at 1000 × *g* and then supernatants were diluted for use in the quantification reaction. Concentrations were determined according to the manufacturer's instructions.

2.4. Quantitative real-time RT-PCR (Q-RT-PCR)

Total RNA samples for Q-RT-PCR were prepared as previously described [9,20]. Quantitative measurements of osteoblast and adipocyte differentiation markers in each cDNA sample were carried out using the Assays-on-Demand™ Gene Expression Kits (Applied Biosystems, Foster City, CA, USA). The cDNA samples were analyzed for adiponectin (Hs00605917_m1), alkaline phosphate (Hs01029144_m1), IL-6 (Hs00985639_m1), leptin (Hs00174877_m1), osteoprotegerin (Hs00171068_m1), and secreted phosphoprotein 1 (Hs00959010_m1). In parallel, relative expression of glyceraldehyde-3-phosphate dehydrogenase, (GAPDH, 4333764F) was measured for data normalization with the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Quantification of relative gene expression levels was determined using equations from a mathematical model developed by Pfaffl [22].

2.5. Western blotting

Western blotting was performed previously described [20]. Phosphorylated STAT3 was measured with anti-phospho-STAT3 antibody (Y705, D3A7, Cell Signaling, Danvers, MA, USA). For detecting STAT3 total protein in hBM-MSCs, anti-STAT3 antibody (79D7, Cell Signaling) was used. β-Actin (Santa Cruz Biotechnology, Santa Barbara, CA, USA) was used as a loading control.

2.6. Statistical analysis

Statistical analyses were performed with MINITAB® software (Minitab Inc. State College, PA, USA). To calculate a *P* value for comparisons between two samples, statistical analyses were performed using Student's *t*-test. For multiple comparisons, data were analyzed using one-way analysis of variance (ANOVA) and then post-hoc tests. The threshold of significance was set at $P < 0.05$.

3. Results

3.1. Leptin synthesis was not correlated to the level of adipogenesis in hBM-MSCs

To study the role of endogenously produced leptin during adipogenesis in the reciprocial regulation of hBM-MSC differentiation, both leptin and adiponectin secretion profiles were systematically investigated by treating hBM-MSCs with various combinations of differentiation inducing factors (Fig. 1). In combinatorial treatments of adipogenic differentiation inducing factors such as dexamethasone, insulin, IBMX and troglitazone, three cell culture conditions including both dexamethasone and IBMX showed significant increases in adipogenesis of hBM-MSCs measured by ORO staining (Fig. 1A). In ELISA, significant up-regulation of adiponectin production was observed in five cell culture conditions containing dexamethasone (Fig. 1B). Most importantly, dexamethasone highly up-regulated leptin secretion

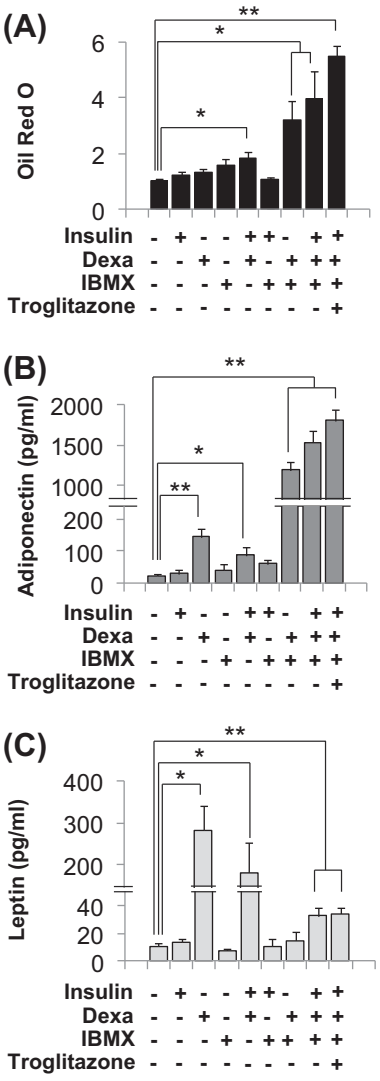

Fig. 1. Systemic analysis of adipogenesis in hBM-MSCs. Adipogenesis was evaluated by treating BM-MSCs with various combinations of adipogenesis inducing molecules, dexamethasone, insulin, IBMX, and troglitazone. To measure adipogenesis, Oil Red O (ORO) staining and quantification was performed (A) at the 14th day in culture. ELISA was performed to measure levels of adiponectin (B) and leptin (C) accumulated in cell culture supernatants for 48 h after the last medium exchange on the 12th day in culture. Results are the mean ± SD of three measurements using independent hBM-MSCs from three different donors ($n = 3$). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

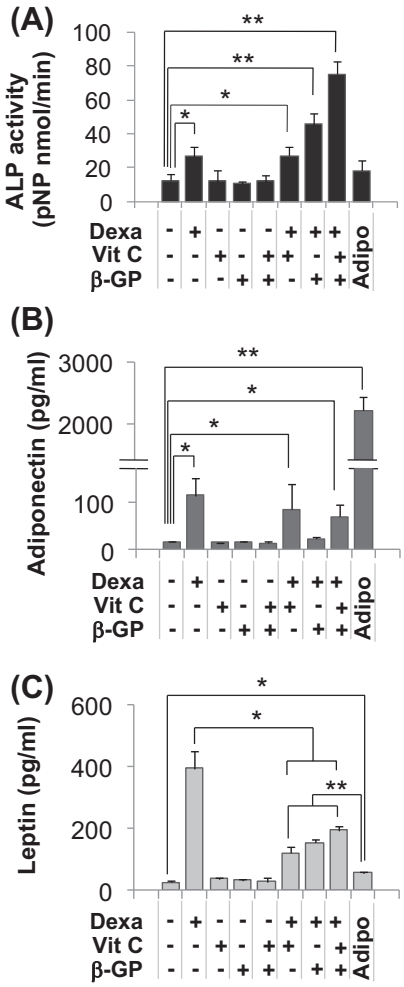

Fig. 2. Systemic analysis of osteogenesis in hBM-MSCs. Osteogenesis was evaluated by treating BM-MSCs with various combinations of osteogenesis inducing molecules, dexamethasone, vitamin C (Vitamin C), and β-glycerol phosphate (β-GP). In parallel cell cultures, adipogenesis was induced with dexamethasone, insulin, IBMX, and troglitazone (Adipo) for the comparison between adipogenesis and osteogenesis. Alkaline phosphatase (ALP) activity was measured to determine osteogenesis in hBM-MSCs (D) at the 14th day in culture. Adiponectin (E) and leptin (F) accumulated in cell culture supernatants for 48 h after the last medium exchange were also evaluated. Results are the mean ± SD of three measurements using independent hBM-MSCs from three different donors ($n = 3$). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

in hBM-MSCs (Fig. 1C). When IBMX, an inhibitor of cellular cyclic AMP phosphodiesterases, was included, leptin production was significantly decreased compared to a single dexamethasone treatment in hBM-MSCs. In contrast, adiponectin levels in the conditions containing IBMX were higher than the condition treated with dexamethasone only (Fig. 1B and C).

3.2. Leptin production during osteogenesis was higher than during adipogenesis in hBM-MSCs

In the combinatorial treatments of osteoblast differentiation inducing factors, four conditions with dexamethasone showed significantly up-regulated alkaline phosphatase (ALP) activity in hBM-MSCs (Fig. 2A). ELISA results showed that adiponectin production was significantly higher during osteogenesis in hBM-MSCs compared to control (Fig. 2B). As consistent with Fig. 1C, leptin production in hBM-MSCs was significantly higher in all dexamethasone positive conditions than in control, and its level was the highest in the single dexamethasone treatment condition, suggesting a pivotal role for dexamethasone in the up-regulation of leptin (Fig. 2C). Interestingly, protein production of leptin in the osteogenic condition was significantly higher than in the adipogenic one.

In Q-RT-PCR, the mRNA level of leptin during osteogenesis was also significantly higher than during adipogenesis in hBM-MSCs, as consistent with protein expression data (Fig. 3A). SPP1, IL-6, and adiponectin levels were also measured in parallel by Q-RT-PCR to evaluate the degree of differentiation for both adipogenesis and osteogenesis in hBM-MSCs (Fig. 3). The mRNA level of adiponectin, a representative adipokine, was significantly up-regulated in the adipocyte differentiation condition. Interestingly, levels of adiponectin gene transcription in both the single dexamethasone and the osteogenic conditions were also increased compared to control (Fig. 3B). Adiponectin production in osteogenesis could be

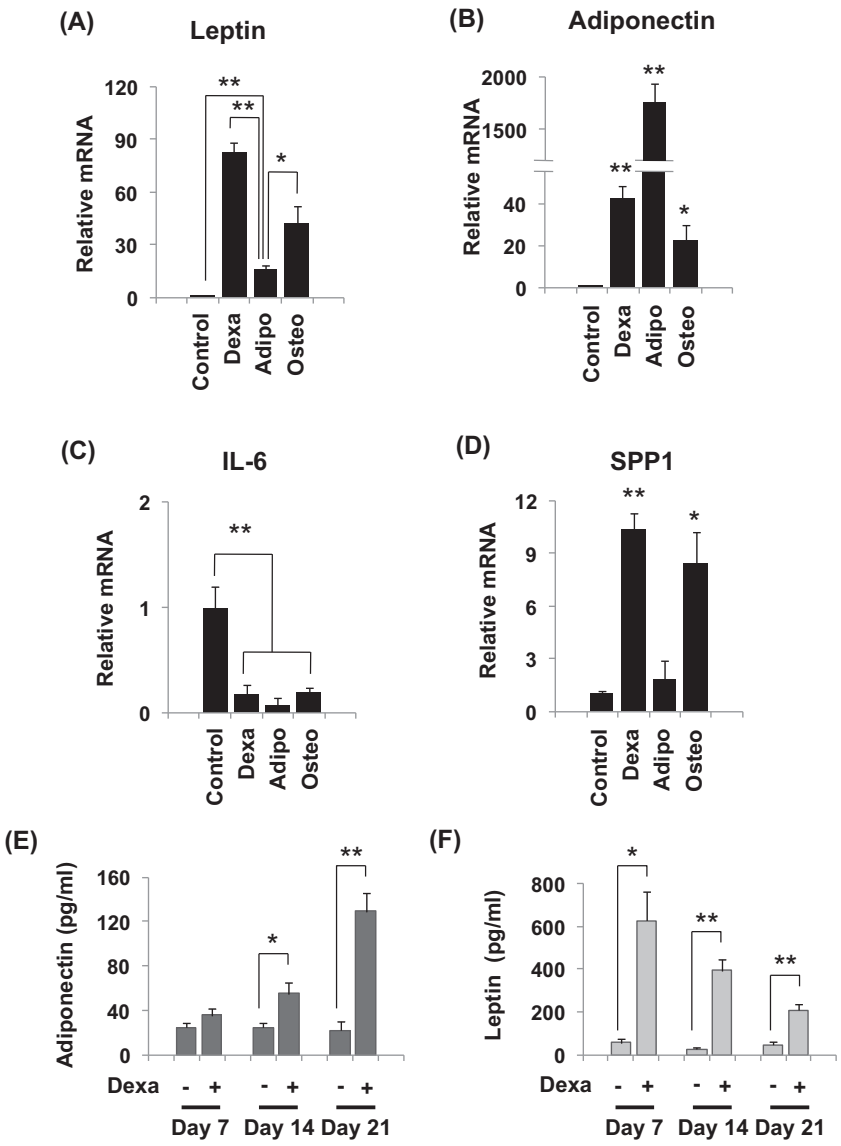

Fig. 3. Validation of leptin production with quantitative real-time RT-PCR (Q-RT-PCR). hBM-MSCs were differentiated in the presence of dexamethasone, the complete adipogenic differentiation condition (Adipo), and the complete osteogenic condition (Osteo) as described in materials and methods. At 14 days in culture, total RNA samples were extracted and Q-RT-PCR was performed for (A) leptin, (B) adiponectin, (C) IL-6, and (D) SPP1. GAPDH was used as an internal control for Q-RT-PCR standardization. To determine the production of adiponectin (E) and leptin (F) on 7th, 14th, and 21st days in hBM-MSC culture in the presence of 100 nM dexamethasone (Dexa), ELISA was performed to measure adiponectin and leptin accumulated in culture supernatants 48 h after medium exchange on the 5th, 12th and 19th days in culture, respectively. Values represent the mean expression ± standard deviation (SD) ($n = 3$). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

interpreted as the activation of heterogeneous differentiation lineage pathways in hBM-MSCs when treated with the osteogenic medium which consisted of dexamethasone, vitamin C, and β-glycerol phosphate. In fact, when hBM-MSCs were cultured over 3 weeks in the presence of dexamethasone alone, a small fraction of hBM-MSCs started to show an adipocyte phenotype with intracellular lipid droplets (data not shown), which was correlated to the increased adiponectin production in the long term culture (Fig. 3E). Interestingly, the levels of adiponectin production in long term dexamethasone treatment showed an inverse relationship with the levels of leptin production in hBM-MSCs (Fig. 3F).

3.3. Leptin promoted osteogenesis in hBM-MSCs while it inhibited adipogenesis

High affinity leptin receptors are expressed in hBM-MSCs [23]. It was reported that leptin promotes osteogenesis and inhibits adipogenesis in immortalized hMS2-12 cells [15]. In this study, it was determined whether leptin can affect the differentiation of primary hBM-MSCs in culture conditions. When leptin was added to the complete osteogenic medium in hBM-MSCs, leptin did not synergistically enhance osteoblast differentiation determined by measuring ALP activity and osteoprotegerin (OPG, also known as TNFRSF11B) production (Fig. 4). Therefore, unlike immortalized hMS2-12 cells, leptin did not synergistically increase osteogenesis in primary hBM-MSC cultures (Fig. 4). However, a single leptin treatment in hBM-MSCs slightly increased ALP activity and significantly up-regulated OPG protein production compared with control, implying a role for leptin in promoting osteogenesis (Fig. 4A and B). However, leptin significantly inhibited adipogenesis in hBM-MSCs in a concentration dependent manner, measured by ORO staining and adiponectin production (Fig. 4C–E). Because the STAT3 pathway is involved in leptin receptor signaling [24],

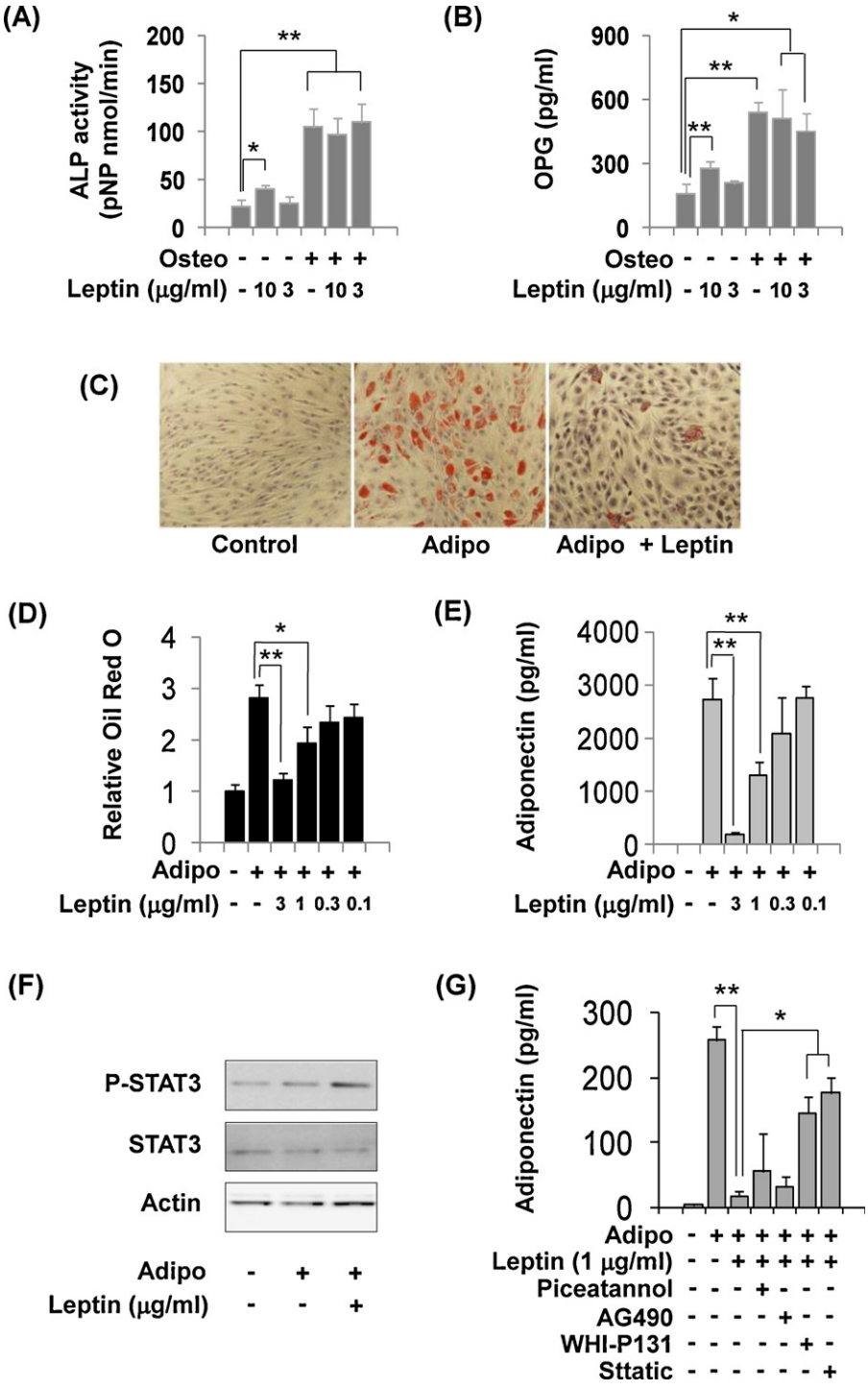

Fig. 4. Effects of leptin on osteogenesis and adipogenesis in hBM-MSCs. hBM-MSCs were cultured in 24 well plates for evaluating adipogenesis and in 6 well plates for testing osteogenesis. Leptin was co-treated in hBM-MSCs with osteogenic differentiation medium, consisting of dexamethasone, vitamin C, and β-GP (Osteo). Media were changed every second or third day in culture. ALP activity was measured to determine osteogenesis in hBM-MSCs at the 21st day after induction of differentiation in culture (A). OPG levels accumulated in cell culture supernatants for 48 h after the last medium exchange were evaluated (B). Leptin and adipogenic medium (Adipo) were used to co-treat hBM-MSCs for 14 days. Fourteen days after adipogenic stimulation, lipid droplets in adipocytes were stained with ORO. To microscopically visualize the nucleus of differentiated cells, hBM-MSCs were counterstained with hematoxylin reagent (C). To quantify these results, the stained ORO was dissolved in isopropyl alcohol, the level of ORO staining was quantified at 500 nm using a spectrometer (D). In parallel, ELISA was performed to measure adiponectin accumulated in cell culture supernatants for 48 h after the last medium exchange (E). Activation of STAT3 signaling was confirmed by Western blotting. Phosphorylated STAT3 was measured with anti-phospho-STAT3 antibody (Y705) (F). A variety of JAK inhibitors (Piceatannol, AG490, and WHI-P131) and a STAT3 inhibitor (Stattic) were used to co-treate hBM-MSCs after the induction of adipogenesis (G). Results are the mean ± SD of three measurements using independent hBM-MSCs from three different donors ($n = 3$). *$P \leq 0.05$ and **$P \leq 0.01$.

we confirmed that leptin increased STAT3 phosphorylation during adipogenesis in hBM-MSCs (Fig. 4F). When Janus kinase (JAK) inhibitors and a STAT3 inhibitor were co-treated with leptin, the leptin-induced down-regulation of adiponectin secretion during adipogenesis was significantly recovered by treatment with WHI-P131, a JAK3 inhibitor, or Stattic, a STAT3 inhibitor (Fig. 4G). The JAK1 inhibitor piceatannol and the JAK2 inhibitor AG490 did not affect the leptin-induced down-regulation

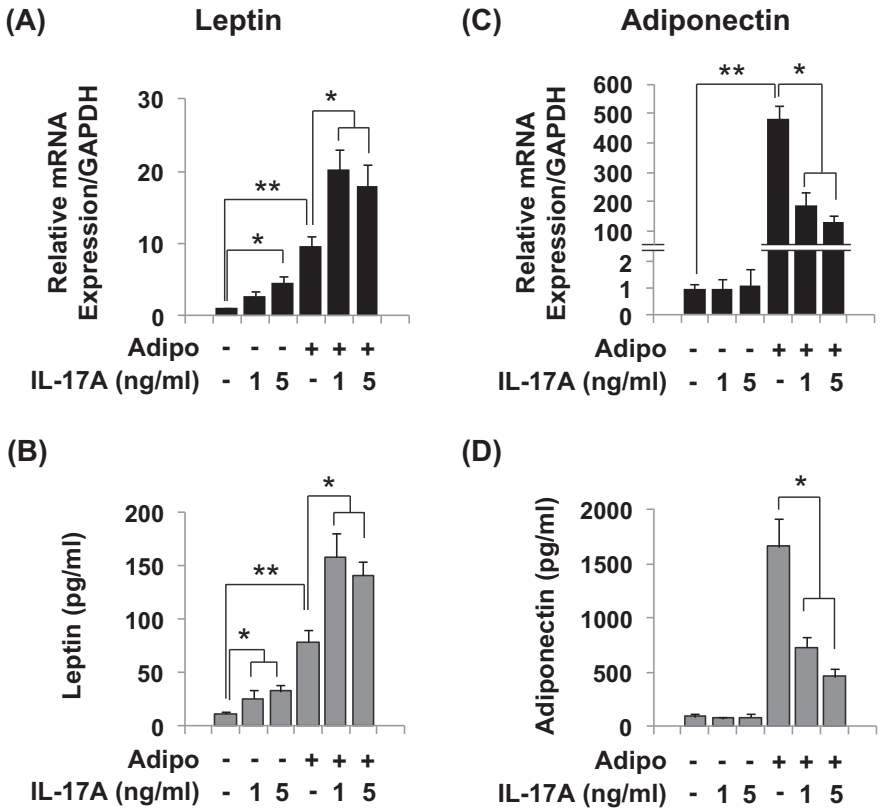

Fig. 5. Effects of IL-17A on the levels of leptin and adiponectin during adipogenesis in hBM-MSCs. hBM-MSCs were cultured in 24 well plates. When cells were confluent, IL-17A was used to treat hBM-MSCs or BM-MSCs in the presence of the complete adipogenic medium (Adipo). After treatment with IL-17A, every two or three days, the media were exchanged. At the 14th day in culture, total RNA samples were extracted and Q-RT-PCR was performed for (A) leptin and (B) adiponectin. GAPDH was used as an internal control for Q-RT-PCR standardization. ELISA was performed to measure levels of leptin (C) and adiponectin (D) accumulated in cell culture supernatants for 48 h after the last medium exchange. Values represent mean ± SD ($n = 3$). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

of adiponectin production during adipogenesis in hBM-MSCs. Therefore, JAK3/STAT3 activation is involved in the leptin-induced inhibition of adipogenesis. Because leptin directly decreased adipogenesis in hBM-MSCs (Fig. 4), there may be inverse relationship between adipogenesis and leptin production in hBM-MSCs in response to dexamethasone containing conditions.

3.4. IL-17A increased leptin production in hBM-MSCs

IL-17A affects adipogenesis and osteogenesis of hBM-MSCs in opposite directions [10–12]. Since IL-17A plays a role in the pathophysiology of human metabolic diseases, it was next evaluated whether the inverse effects of IL-17A on adipogenesis and osteogenesis are related to leptin production. IL-17A significantly increased gene transcription and protein expression of leptin in hBM-MSCs (Fig. 5). During adipogenesis, IL-17A increased leptin production while simultaneously decreasing adiponectin production (Fig. 5). During osteogenesis in hBM-MSCs, IL-17A at low concentrations increased both leptin production and ALP activity (Fig. 6). As the concentration of IL-17A was increased above 50 ng/ml, neither leptin production nor ALP activity was changed compared with control (Fig. 6A and B). As previously reported, there was no cytotoxic effect of 50 ng/ml IL-17A [10]. It suggests that it is important to treat hBM-MSCs with an optimal IL-17A concentration to study the promoting effect of IL-17A on osteogenesis. Importantly, the degree of synergistic enhancement of osteogenesis by IL-17A was correlated to levels of leptin production (Fig. 6).

3.5. Neutralizing leptin by anti-leptin antibody treatments increased adiponectin production during osteogenesis in hBM-MSCs

To verify that the IL-17A-induced leptin production was associated with the synergistic promotion of osteogenesis and also with the inhibition of adipogenesis, leptin was neutralized by treating hBM-MSCs with anti-leptin antibody (Fig. 7). During osteogenesis, ALP activities were unchanged by treating hBM-MSCs with anti-leptin antibody (Fig. 7A). However, anti-leptin antibody treatment resulted in significant adiponectin production during osteogenesis (Fig. 7B). In fact, it was microscopically observed that long term treatment of the anti-leptin antibody in the complete osteogenic condition increased cell populations with lipid droplets (data not shown). In addition, anti-leptin antibody was treated to determine whether the IL-17A-induced leptin production was associated with the inhibitory effect of IL-17A on adipogenesis and the promoting effect on osteogenesis of hBM-MSCs. The anti-leptin antibody treatment did not affect the IL-17A-induced synergistic enhancement of osteogenesis determined by ALP activities and OPG production (Fig. 7C and D). However, anti-leptin antibody treatment partially attenuated the IL-17A induced-inhibition of adipogenesis in hBM-MSCs (Fig. 7E and F). Taken together, the up-regulation of leptin by dexamethasone and/or IL-17A in hBM-MSCs plays a role in suppressing adipogenesis and thereby indirectly promotes osteogenesis.

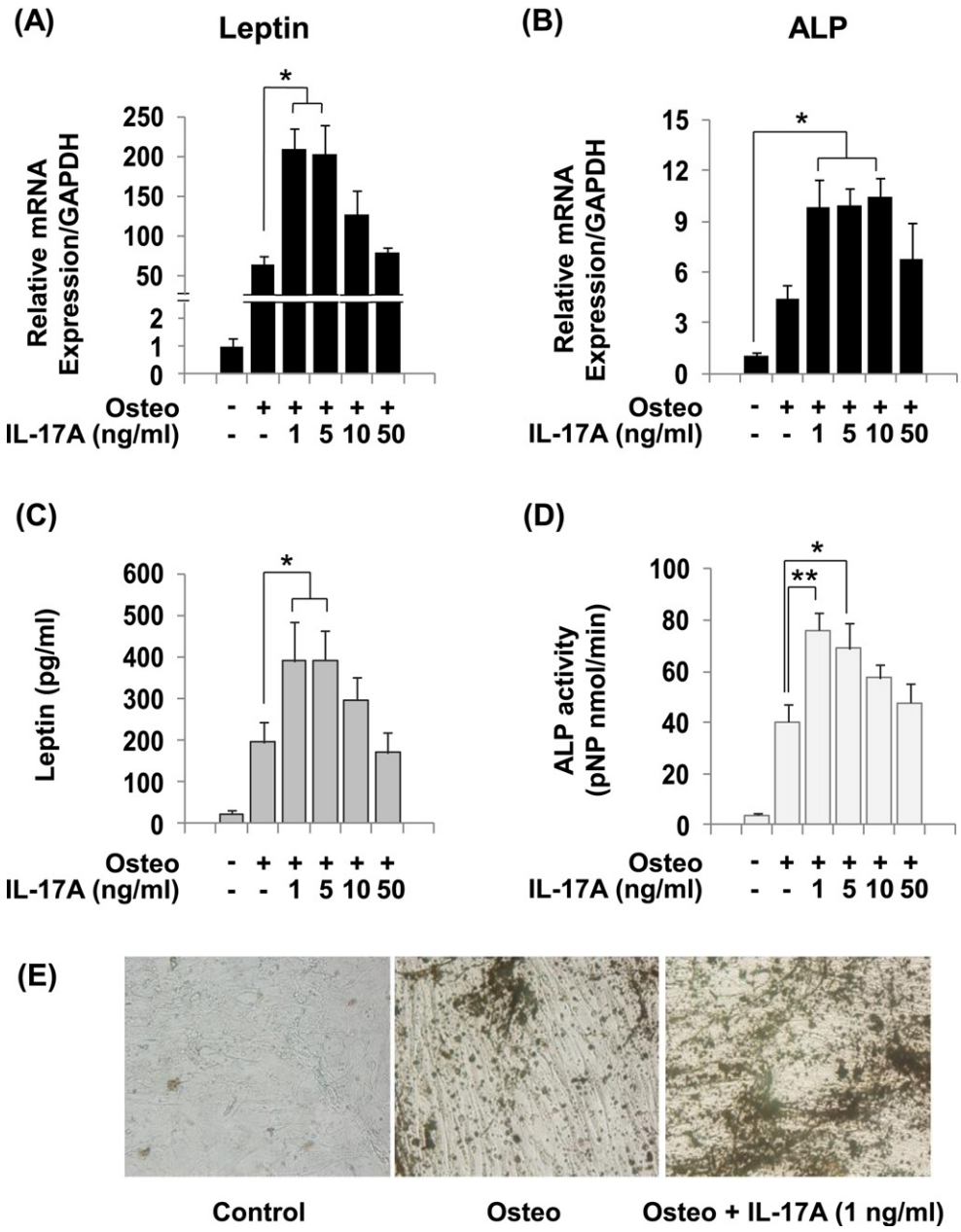

Fig. 6. Effects of IL-17A on levels of leptin and adiponectin during osteogenesis in hBM-MSCs. hBM-MSCs were cultured in 6 well plates. When confluent, IL-17A was added to treat hBM-MSCs or BM-MSCs in the presence of the complete osteogenic condition (Osteo). After treating with IL-17A, every two or three days, media were exchanged. At the 21st day in culture, total RNA samples were extracted and Q-RT-PCR was performed for (A) leptin and (B) alkaline phosphatase (ALP). GAPDH was used as an internal control for Q-RT-PCR standardization. ELISA was performed to measure levels of leptin (C) accumulated in cell culture supernatants for 48 h after the last medium exchange. ALP activity was measured in hBM-MSCs (D). Relative alkaline phosphatase activity was normalized to protein levels and expressed as the molar amount of *p*-nitrophenol produced per 1 min. Von Kossa's staining was performed to measure mineralization (E). Values represent mean ± SD ($n = 3$). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

4. Discussion

Leptin, an adipokine central to regulation of appetite and energy metabolism, was highly up-regulated in hBM-MSCs by a single dexamethasone treatment and by various differentiation inducing conditions including dexamethasone (Figs. 1 and 2). Interestingly, leptin production during osteogenesis in hBM-MSCs was even greater than that during adipogenesis (Figs. 1 and 2). A recent study showed that leptin and its cellular receptors were highly detected in osteoblasts isolated from osteoarthritis patients [25], suggesting that extra-adipose tissues such as osteoblasts can produce leptin in pathologic conditions like osteoarthritis. In addition, dexamethasone-induced leptin production in hBM-MSCs can help us understand the metabolic outcomes of corticosteroid side effects. Because hMSCs are present in the perivascular region throughout virtually all post-natal tissues [26], further studies to test this postulate will be required to address this question [19].

In immortalized hMS2-12 cells, leptin synergistically promoted osteogenesis [15]. Although a single leptin treatment in primary hBM-MSCs increased ALP activity and OPG production, the addition of exogenous leptin did not synergistically promote osteogenesis in hBM-MSCs under the complete osteogenic condition (Fig. 4). Because significant amount of leptin was endogenously produced during osteogenesis in hBM-MSCs (Fig. 2), the leptin receptor signaling pathway can be desensitized in the consistent presence of leptin during osteogenesis in hBM-MSCs

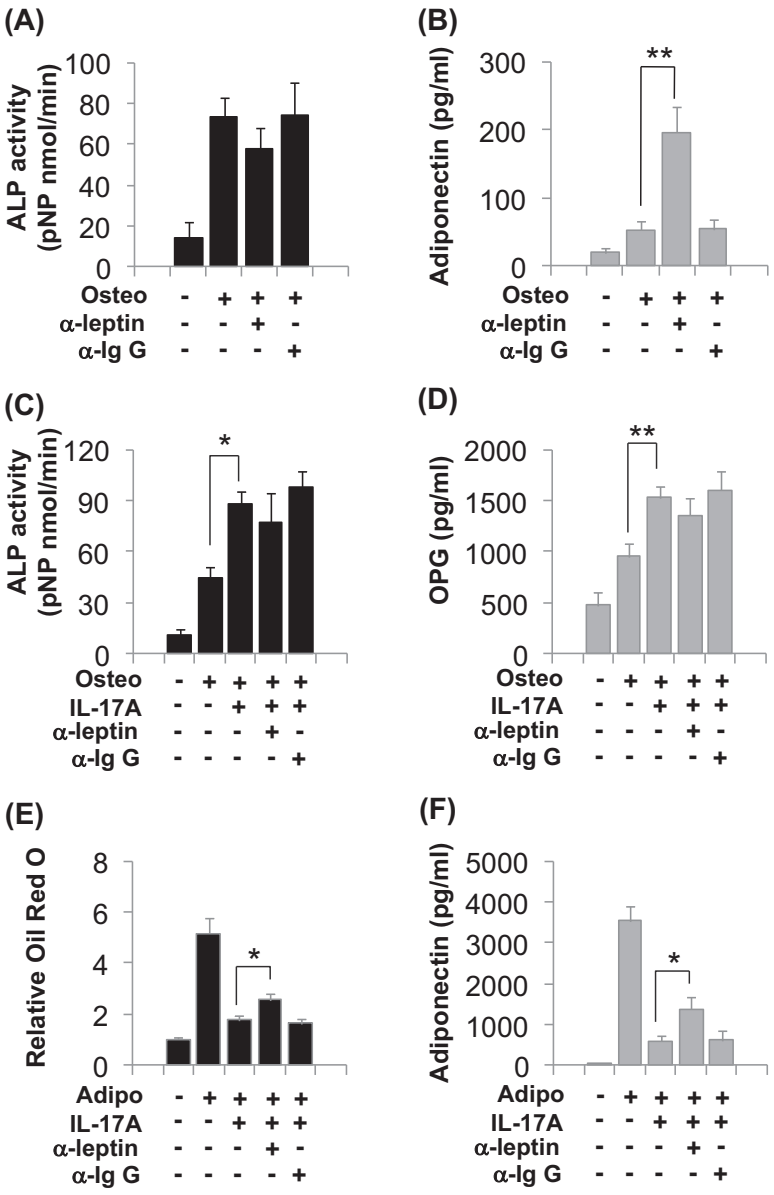

Fig. 7. Roles of leptin in the reciprocal effects of IL-17A on osteogenesis and adipogenesis in hBM-MSCs. During osteogenesis in hBM-MSCs cultured in 6 well plates, anti-leptin antibody (α-leptin) or Ig G was added. After 21 days in culture, ALP activity was measured in hBM-MSCs (A) and simultaneously ELISA was used to measure adiponectin levels accumulated in the culture supernatant for 48 h after the last medium exchange (B). To determine roles for IL-17A-induced leptin in osteogenesis, ALP activity (C) and OPG levels were measured on the 21st day after inducing osteogenesis in hBM-MSCs. To evaluate the effects of the IL-17A-induced leptin on adipocyte differentiation, ORO staining was performed at the day 14 after inducing adipogenesis (E) and adiponectin levels in the culture supernatant were measured (F). Values represent mean ± SD ($n = 3$). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

[23,24]. Therefore, exogenously treated leptin may not be able to fully activate leptin receptor signaling because it was already desensitized by endogenously produced leptin in hBM-MSCs, resulting in no synergy for osteogenesis by exogenous leptin treatment (Fig. 4). Differences in leptin-induced synergistic enhancement of osteogenesis between hBM-MSCs and hMS2-12 cells can be explained by the different leptin expression profiles of those cells. Recent reports showed that exogenous leptin can play a role as an osteogenic factor in leptin deficient *ob*/*ob* mice [27,28]. Although leptin was an osteogenic factor in leptin deficient animals, when leptin levels in circulation recovered to normal, there was no significant promoting effect on bone formation by additional leptin administration above the physiological level [27,29]. Therefore, leptin can contribute to promote osteogenesis in the deficient condition for bone formation but, in the complete differentiation condition, the effect of leptin may be limited by desensitization of leptin receptor signaling.

In hBM-MSCs, leptin can indirectly contribute to the osteogenesis by suppressing adipogenesis. Leptin significantly inhibited adipogenesis via JAK3/STAT3 signaling in hBM-MSCs (Fig. 4G). It was reported that the effect of leptin on osteogenesis was antagonized by both a JAK1 inhibitor AG490 and a JAK2 inhibitor piceatannol [24]. Therefore, leptin receptor signaling pathways affecting osteogenesis and adipogenesis in hBM-MSCs may be different in terms of JAK, although it shares STAT3 as a signaling molecule. Further study will be required to clarify the difference between leptin-induced JAK/STAT signaling between osteogenesis and adipogenesis. Importantly, anti-leptin antibody treatment during osteogenesis of hBM-MSCs increased adiponectin production in culture (Fig. 7), suggesting a role for increased leptin levels

in suppressing adipogenesis. In combinatorial treatments with molecules that induce adipogenesis, it was found that the cAMP phosphodiesterase inhibitor IBMX played a key role not only in the down-regulation of leptin production (Fig. 1C). Leptin receptor activation inhibits the glucagon-dependent cAMP elevation in rat hepatocytes [30], suggesting the existence of negative feedback mechanisms between leptin and cAMP-related signaling pathways in hBM-MSCs. For example, cAMP regulated signaling molecules like protein kinase A may regulate leptin production in hBM-MSCs in response to endogenous corticosteroids. Because leptin resistance or abnormal leptin production is related to metabolic diseases [31,32], it will be interesting to study cellular mechanisms that regulate leptin production in hBM-MSCs in the presence of various hormones and cytokines associated with disease progression.

In contrast to leptin, IL-17A showed synergistic enhancement of osteogenesis in hBM-MSCs (Fig. 6). Unlike leptin, there was no endogenous IL-17A production by hBM-MSCs. Therefore, the IL-17A receptor signaling pathway in hBM-MSCs may fully respond to IL-17A. IL-17A inhibited adipogenesis in hBM-MSCs [10]. Interestingly, IL-17A significantly increased leptin production during adipogenesis while it decreased adiponectin production (Fig. 4). Anti-leptin antibody treatments attenuated the effects of IL-17A on adipogenesis in hBM-MSCs (Fig. 6), suggesting that leptin contributed to the IL-17A-dependent inhibition of adipogenesis. As mentioned earlier, IL-17A is associated with metabolic diseases like obesity and type II diabetes [12–14]. Diet-induced hyperleptinemia correlates to tissue resistance of both insulin and leptin [31]. Taken together, it is possible that the increased leptin production in both adipocytes and undifferentiated hBM-MSCs that is induced by IL-17A may be involved in the progression of insulin resistance associated with type II diabetes.

Patients with type II diabetes are a high risk population for hip bone fracture [1–3]. Paradoxically, bone mineral density in patients with type II diabetes is higher than in the normal population [2,3]. Currently, it is still controversial why bone fracture incidence is higher in type II diabetic patients in spite of the high bone mineral density. It was reported that circulating Th17 cells were increased in blood from type II diabetes patients [14]. It is possible that circulating Th17 cells in type II diabetic blood may have an impact on bone mineral density. Leptin also has ro-osteogenic effects on leptin deficient animals [30] and a single leptin treatment promoted osteogenesis in hBM-MSCs (Fig. 3). Therefore, the increased bone mineral density in type II diabetes may be explained by IL-17A and leptin produced in hMSCs stimulated by IL-17A. To elucidate why bone fracture incidence is increased in type II diabetes, it will be necessary to study whether the quality of bone formation is affected by pro-osteogenic cytokines like IL-17A and leptin, especially whether they are up-regulated in the metabolic diseases. To test this postulate on the relationship between type II diabetes and increased bone fractures, studies are on-going to reveal how IL-17A-dependent enhancement of osteogenesis in hBM-MSCs is qualitatively different from normal osteogenesis in the context of cross-talk with leptin receptor signaling pathways.

In summary, leptin production during osteogenesis was significantly higher than that during adipogenesis, implying that both hBM-MSCs and osteoblasts can produce leptin. Leptin inhibited adipogenesis and promoted osteogenesis in hBM-MSCs. IL-17A significantly increased leptin production in hBM-MSCs. Anti-leptin antibody treatment partially antagonized the IL-17A dependent inhibition of adipogenesis in hBM-MSCs, suggesting a role of leptin in mediating the inverse regulation of IL-17A on osteogenesis and adipogenesis in hBM-MSCs. Due to the association of Th17 cell responses with type II diabetes, IL-17A-dependent leptin production may contribute to the progression of insulin resistance. Since both IL-17A and leptin can promote osteogenesis in hBM-MSCs, the IL-17A-induced leptin production may provide a key clue to understand the relationship between type II diabetes and increased bone fractures.

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